High-Throughput Screening of Antimalarials via Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

by

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LIST OF ABBREVIATIONS

DESI……..……Desorption Electrospray Ionization

ESI…….……...Electrospray Ionization

GC……..……..Gas Chromatography

HPLC……...……High-Pressure Liquid Chromatography

LC……Liquid Chromatography

*m/*z.....Mass-to-charge Ratio

MRM……Multiple Reaction Monitoring

MS……..…….Mass Spectrometry

MS/MS or MS^2……Tandem Mass Spectrometry

NIR……...……Near Infrared

SRM……..…Single Reaction Monitoring

TLC…….….Thin-Layer Chromatography

SUMMARY

The counterfeiting of pharmaceuticals, especially antimalarials, is a well-recognized and growing public health problem. There have been an alarming number of reports of counterfeit antimalarials throughout the world and insufficient regulations and high demand for these very costly pharmaceuticals continues to fuel counterfeiting activity. Thus there is an urgent need for a rapid and sensitive authentication and screening tool for multiple antimalarials. While many methods have been developed using HPLC, MS, or LC-MS to screen individual antimalarials, no methods are available for the analysis of multiple antimalarial drugs within a single run. In this study, Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) with multiple reaction monitoring (MRM) was used as a selective and rapid way to screen ten common antimalarials. The compounds were first individually analyzed using electrospray ionization mass-spectrometry (ESI-MS). Following this, a corresponding MS/MS spectrum was obtained to enable selection of the optimal unimolecular decay fragmentation ("transition") of each antimalarial. Finally, these single reaction monitoring (SRM) transitions were combined into a method that utilizes HPLC separation followed by multiple reaction monitoring (MRM) in an ion trap mass analyzer allowing for sequential screening of a mixture of these compounds within a single LC-MS/MS run. This method has both pharmaceutical and medical applications with the capability of providing drug quality control measurements and the detection of many drugs and their metabolites in biological samples.

CHAPTER 1: INTRODUCTION

Malaria is a disease caused by the protozoan parasite *Plasmodium* which is transmitted from person to person through bites from infected female *Anopheles* mosquitoes. The disease has grown into a significant global health crisis, and the crisis is still present today. Between 400 and 900 million cases of malaria are reported every year causing an average of 1 to 3 million deaths[1]. Since no vaccine is available for malaria, all treatments and preventatives come in the form of prescription drugs, called antimalarials. There are numerous antimalarials available, and often treatments will include a combination of two drugs. However, the high cost of and demand for antimalarials, which typically do not have a generic form, drive counterfeiting activity which severely impedes the prevention and treatment of the disease[2, 3].

Counterfeit antimalarials come in different forms. They can contain the wrong ingredient(s), not contain any active ingredient(s), or have insufficient levels of active ingredient(s). Counterfeit antimalarial drugs have been found in Africa, Asia and South America[4], and very sophisticated fakes have been reported in Thailand, Vietnam, and Cambodia[5]. A survey from Cambodia showed 60% of antimalarials labeled as mefloquine contained ineffective and cheaper sulphadoxine-pyrimethamine[6, 7] and a study in Southeast Asia revealed that 38% of 104 artesunate samples gathered were counterfeit[8]. The widespread nature of this problem has provided motivation for companies and academic research groups to develop new methods and technologies attempting to combat the persistence of counterfeit antimalarials.

CHAPTER 2: LITERATURE REVIEW

Several different physical and chemical tests can be used for the analysis of antimalarials. There are a variety of chemical tests available which range from testing for the presence of active ingredient in a tablet to quantifying the amount of antimalarial present in the bloodstream. The tests can also vary in complexity ranging from simple field tests to advanced analytical techniques[9]. Where high tech lab equipment may be unavailable, some field tests including thin-layer chromatography (TLC) [10], colorimetry[8, 11], and a one-step fluorescence assay[12] have been developed. While these are simple, inexpensive, and easy to perform, more in depth information on an antimalarial is typically needed or beneficial seeing that identification of a counterfeit antimalarial is only the first step in attacking the problem. Information on the type of counterfeiting and the drug's origin can only be determined with more advanced techniques.

Advanced screening techniques have the ability to provide crucial chemical information. These methods are often much more sophisticated than simple chemical techniques and are performed with highly developed laboratory equipment. Some advanced techniques that in recent years have been applied to antimalarials include liquid chromatography (LC) and high-performance liquid chromatography (HPLC) [4, 13, 21], gas chromatography (GC)[10], UV-Vis spectroscopy[14], near-infrared (NIR) spectroscopy[11, 14-16], Raman spectroscopy[17], mass spectrometry (MS)[18, 19], and liquid chromatography-mass spectrometry (LC-MS) [11, 20, 23-27].

2.1 High Performance Liquid Chromatography (HPLC)

HPLC is a widely used analytical technique that is used to separate, purify, or quantify components in a complex mixture based on component properties such as polarity, ionic charge, or size. Identification of a compound with HPLC requires the use of a detector coupled to the HPLC system. Therefore an HPLC detection system must first perform a separation assay that flows into the detector.

HPLC methods have been applied to antimalarials, including the separation of impurities from a novel antimalarial drug 8-aminoquinolone[21], and the separation of three antimalarial drugs, chloroquine, quinine, and mefloquine[4]. Additionally, Dr. Harparkash Kaur of the London School of Hygiene and Tropical Medicine recently developed an HPLC method to separate a number of antimalarials with UV-Vis detection[22]. While this method can separate a mixture of many antimalarials, UV-Vis detection is not as sensitive or selective as other detection options such as mass spectrometry.

2.2 Mass Spectrometry (MS)

Perhaps one of the most powerful and sensitive screening techniques available for antimalarial analysis is mass spectrometry (MS). A wide range of different MS techniques including accurate mass ESI MS, accurate mass tandem MS, and desorption electrospray-ionization (DESI) have been applied to different types of antimalarials[18, 19]. MS has a number of advantages over other analytical methods including high speed and sensitivity. However, like the antimalarial HPLC methods[4, 21], all current antimalarial MS methods share the same limitation - they are designed to analyze a single drug or a small number of antimalarial drugs.

2.3 LC-MS

Liquid chromatography-mass spectrometry (LC-MS) is an analytical technique that couples the separation power of HPLC with the sensitive and selective identification power of mass spectrometry.

Numerous methods using LC-MS have been developed for antimalarials. Individual LC-MS methods have been developed for the analysis of chloroquine[23], piperaquine[24], pyrimethamine and sulfamethoxypyrazine[25], and artesunate and dihydroartemesinin[26] in plasma. Another method has also been developed for analysis of artemisinin from plant extracts[27]. But yet again, a major limitation of these methods is that each has only been developed for one or two antimalarials[23-27].

In summary, although the analysis of a mixture of different antimalarials using LC with UV detection has been demonstrated[22] as has the development of varying LC-MS methods for individual antimalarials[23-27], no work has been published combining these two techniques. While each type of analytical method has unique advantages, one need that no recent HPLC, mass spectrometry, or LC-MS antimalarial method addresses is the simultaneous separation and identification of multiple antimalarial drugs within a single run. Where some of the referenced methods have been applied to a couple of different antimalarials, most have been developed solely for the analysis of a single drug [4, 17-21, 23-27].

Therefore the goal of this project is to determine if the HPLC separation of multiple antimalarials can be successfully combined with tandem MS into one high-

throughput and sensitive screening tool. LC-MS was the technique of choice due to the ability to couple the separation power of HPLC with the highly specific and sensitive identification capabilities of MS.

Due to the large number of different antimalarials on the market world-wide and the high incidences of counterfeiting, a method with these screening capabilities would be of great benefit in the pharmaceutical industry. Furthermore, many people in endemic malaria regions often self-medicate with combinations of antimalarial drugs and the ability to separate and detect these combination therapeutics could be applied to blood plasma samples as well. Ultimately, the ability to test a large number of drugs in a single, sensitive, and large scale assay creates the potential to save time, money, and ultimately save lives.

CHAPTER 3: EXPERIMENTAL

3.1 Standards, Solvents, and Sample Prep

The antimalarials of interest for this project were chosen from those that Dr. Kaur successfully separated with HPLC[22]. The selected antimalarials were: amodiaquine, chloroquine, dapsone, dihydroquinine, mefloquine, primaquine, pyrimethamine, quinidine, quinine, sulfadoxine, and trimethoprim (See Appendix A for a description and structure of each). The solid standard for each antimalarial was purchased from Sigma-Aldrich and was used without any further purification.

Name	CAS #
Amodiaquine	6398-98-7
Chloroquine	50-63-5
Dapsone	80-08-0
Dihydroquinine	522-66-7
Mefloquine	51773-92-3
Primaquine	63-45-6
Pyrimethamine	58-14-0
Quinidine	6151-40-2
Quinine	6119-70-6
Sulfadoxine	2447-57-6
Trimethoprim	738-70-5

Table I: Antimalarial standards purchased from Sigma-Aldrich.

Stock solutions were prepared for each antimalarial in 50/50 (v/v) HPLC grade methanol (Sigma Aldrich, St. Louis, MO) and deionized water (18 MΩ cm^{-1}, Barnstead International, Dubuque, IA). The standard solutions for ESI experiments were prepared in a 50/50 solution (v/v) of methanol (Sigma Aldrich, St. Louis, MO) and deionized water with 0.1 % acetic acid (Sigma Aldrich, St. Louis, MO). The antimalarial mixtures for LC-

MS/MS were prepared in deionized water. All sample solutions were prepared on a daily basis from stock solutions stored at -80º C. A solution that was 5 μM for each antimalarial was used during the optimization of the method while a set of standards (0.1, 0.3, 0.5, and 1.0 μM) was used while calibrating the response of the system. The HPLC system utilized gradient elution of deionized water with 0.1% formic acid (Fisher Scientific, Waltham, MA) and acetonitrile (Sigma Aldrich, St. Louis, MO) with 0.1% formic acid as the solvents. All samples were filtered using a 0.45 μm PTFE membrane filter before analysis.

Human plasma samples were prepared using 100 μL of plasma (Sigma Aldrich, St. Louis, MO) spiked with chloroquine, amodiaquine, or dihydroquinine. The mixture was then vortexed for 5 seconds and sonicated for 10 minutes. Next, the mixture was diluted to 300 μL with acetonitrile, vortexed for 10 seconds, and sonicated for 15 minutes. Samples were then centrifuged at 13,000 g for 30 minutes. The resulting supernatant was pipetted into a clean centrifuge tube and the solvent was evaporated at 45°C for approximately 3 hours. The residue was then reconstituted with 100 μL deionized water. Finally, after the sample was filtered with a 0.45 μm syringe filter it was ready for analysis.

3.2 Experimental Procedures

The LC-MS/MS method development consisted of two main parts:

(1) Analyzing each antimalarial individually using continuous infusion electrospray ionization tandem mass spectrometry (ESI MS/MS) (See Appendix B for ESI description).

(2) Analyzing the mixture of antimalarials using LC-MS/MS.

Each antimalarial standard was first run using ESI MS/MS to identify and select the unimolecular decay fragmentations ("transitions") to be monitored for each drug. An optimized tune file method was then created for each. Following this step the LC-MS/MS experiments could be performed by setting up the method to screen for each fragmentation transition identified in (1) using multiple reaction monitoring (MRM) of a mixture of antimalarials (See Appendix B for MRM description). Further optimization of the method was the next experimental step. The final step was the creation of a calibration curve and subsequent analyses of spiked human plasma samples.

3.3 LC-MS Instrumentation

LC was performed on a LDC Analytical system equipped with a Constametric 3200 solvent delivery system and a GM4000 gradient programmer, specifically assembled for this project. A 2.0 mm ID, 75 mm long Shim-Pack XR-ODS reverse-phased column (Shimadzu, Columbia, MD) was used in all of the experiments. The LC was operated at a flow rate of 500 μL min^{-1} with an injection volume of 20 μL. The binary pump used deionized water with 0.1% formic acid as mobile phase A and a 95% acetonitrile solution with 0.1% formic acid as mobile phase B. The LC gradient started at 20% B and ramped to 100% B in 10 minutes. The system was flushed with 100% mobile phase B following each run for approximately 10 minutes, and allowed to equilibrate to the original mobile phase composition before beginning another run. The HPLC

instrument was coupled to a Thermo LCQ DECA XP+ quadrupolar ion trap mass spectrometer equipped with an electrospray ionization ion source.

3.4 LC-MS/MS Software Set-Up

The LC-MS/MS method used Xcalibur software to set up and queue each run. The run time, individual transitions to monitor (up to 10), and method tune file were all entered under the instrument setup. Next the file name, file path, and instrument setup were entered into a sequence setup, which was then used to begin each run. The software could not interface with the HPLC pump controls, therefore all gradient adjustments were done manually on the GM4000 gradient programmer.

CHAPTER 4: RESULTS

4.1 Identifying Antimalarial SRM Transitions

Each antimalarial was run with ESI (in a 50:50 acetonitrile:water, 0.1% acetic acid solvent system) in positive and negative ion mode. Positive ion mode produced stronger signal and better fragmentation for the majority of the samples; therefore the method was developed using this mode. A mass spectrum was collected for each antimalarial once the precursor ion was identified. This peak was then optimized to obtain the maximum signal intensity (Figure I). After tuning, the precursor peak was then fragmented and a MS/MS spectrum was collected. The percent collision energy was optimized to produce a minimum but still visible precursor peak ion intensity and maximum fragment ion intensity (Figure II). Based upon the unique fragment ions identified for each antimalarial, a corresponding precursor to product ion transition was selected for each antimalarial and the single reaction monitoring (SRM) mass spectrum was then collected for each (Figure III).

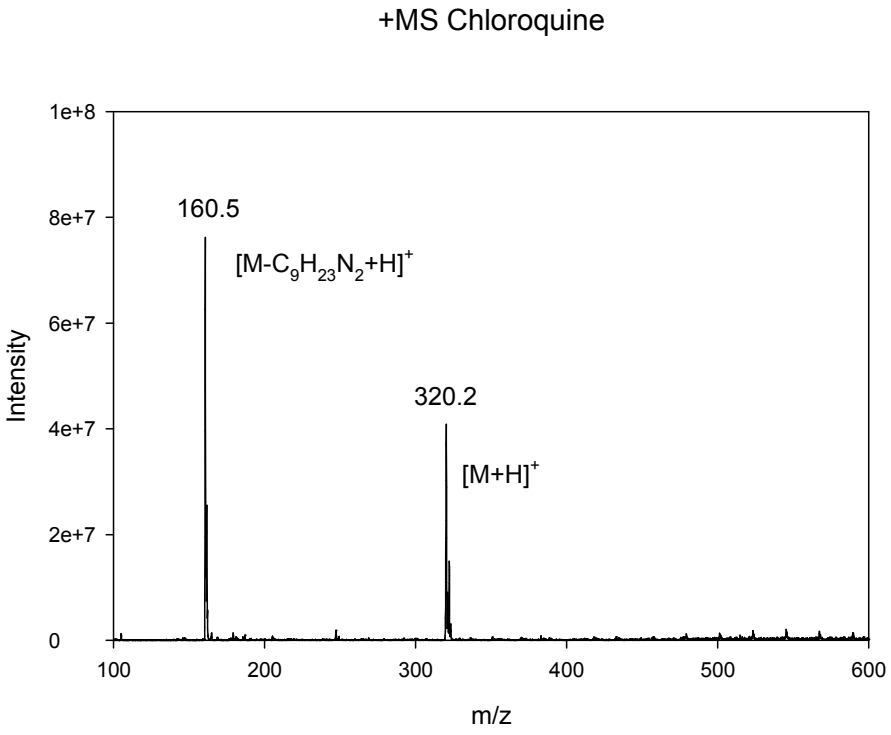


Figure I: The positive mode full mass spectrum for the antimalarial chloroquine. The $[M+H]^+$ peak appears at *m/z* 320.2, and the doubly-charged $[M+2H]^{2+}$ peak appears at *m/z* 160.5.

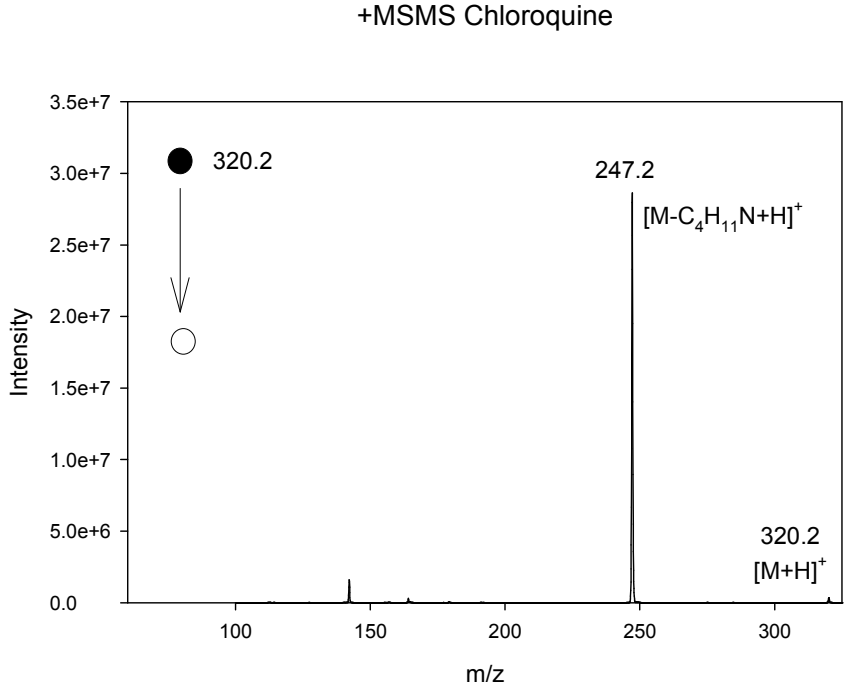


Figure II: The positive mode MS/MS (or MS^2) spectrum for chloroquine showing the predominant fragment peak corresponding to $[M-C_4H_{11}N+H]^+$ at *m/z* 247.2.

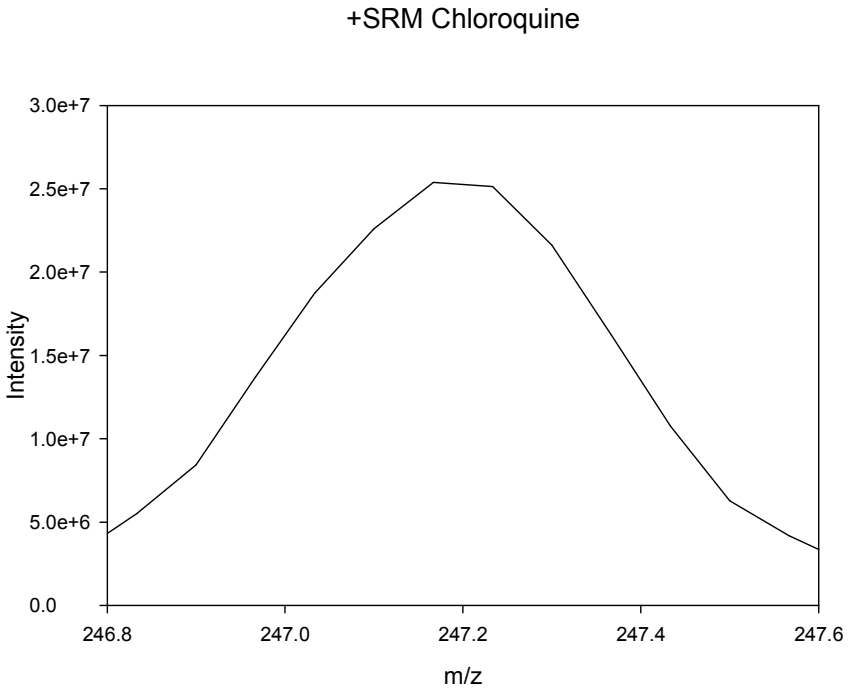


Figure III: The positive mode single reaction monitoring (SRM) spectrum, which monitors only for the product ion at *m/z* 247.2.

4.2 Development of ESI Tune Files for Antimalarials

After identifying the unique SRM transition for each antimalarial, a method tune file was created and saved for each drug. Some major variable conditions and the unique precursor to product SRM transition for each antimalarial are summarized in Table II.

Antimalarial	Capillary Voltage (V)	Capillary Temp (ºC)	Tube Lens (V,sp)	Collision Energy (%)	Isolation Width	Precursor *m/z*	Product *m/z*
Amodiaquine	34.18	300	20	36	1.0	356.1	283.2
Chloroquine	13.98	300	5	36	1.0	320.1	247.2
Dapsone	28.1	299.8	30	36	1.0	249.1	156.0
Dihydroquinine	43.33	300	45	46	1.0	327.3	309.2
Mefloquine	46.47	300.5	45	36	1.0	379.2	361.2
Primaquine	25.03	299.8	0	30	1.0	260.1	243.2
Pyrimethamine	2.85	300.2	25	53	1.0	249.3	233.1
Quinidine	46.33	300	45	42	1.0	325.2	307.2
Quinine	19.03	300.1	15	44	1.0	325.3	307.2
Sulfadoxine	9.07	300.1	10	36	1.2	311.2	156.0
Trimethoprim	2.78	300	-10	40	1.0	291.3	230.1

Table II: A summary of the important variable conditions for each antimalarial with ESI MS/MS.

4.3 HPLC Split Flow Experiment

In order to properly interface the HPLC to the mass spectrometer, a flow splitter was used to reduce the flow entering the mass spectrometer inlet and thus, the split ratio for the splitter valve had to be measured using a split flow experiment. This split flow ratios were determined for incremental turns of the splitter by massing the amount of deionized water (at 22ºC) collected from each line (sample and waste) in a two minute time period. The increments were measured by every half turn until the valve was completely open after 3.5 turns. In the region where the greatest change in flow ratio was observed, additional points were collected to provide a better indication of whether any reproducible intermediate flow ratios would be created. The experiment was performed at several different flow rates, but 0.5 mL/min was chosen as the final flow rate to be used for subsequent experiments, as it was the manufacturer's recommended flow rate for the HPLC column used. No changes in the minimum or maximum sample flow rate through the splitter were observed while changing the overall pump flow rate. The experimental

data in Table III shows the calculated sample flow rate for a split flow experiment at 0.5 mL/min.

# Turns	Waste Flow (mL/min)	Sample Flow (mL/min)
0.0	0.4743	0.0004
0.5	0.4848	0.0003
0.54	0.4089	0.1014
0.58	0.4039	0.1016
0.66	0.4037	0.1011
0.75	0.4105	0.1055
1.0	0.3880	0.0987
1.25	0.4002	0.1051
1.5	0.3947	0.1008
2.0	0.3962	0.1007
2.5	0.3961	0.1028
3.0	0.3992	0.1044
3.5	0.3982	0.1032

Table III: A summary of the waste and sample line flow rates from a split flow ratio experiment performed at 0.5 mL/min.

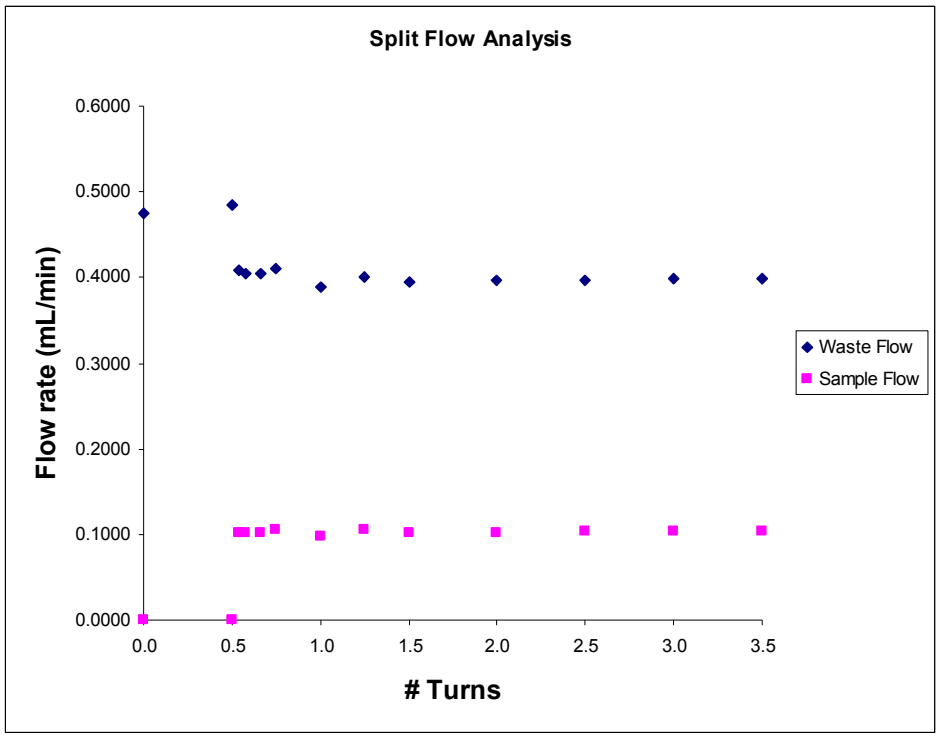


Figure IV: A plot of the sample and waste flow rates determined from the split flow analysis at 0.5 mL/min.

Figure IV shows the flow rates for the sample and waste line based on the numbers of turns the splitter is open at 0.5 mL/min. Since the max sample flow rate of the splitter is about 0.1 mL/min and this flow will work properly at the inlet of the ESI source on the mass spectrometer, all subsequent LC-MS/MS experiments were operated with the splitter fully opened.

4.4 Optimization of LC-MS/MS Method

Once the individual ESI tune files and the split flow experiment were completed, the initial LC-MS/MS trials began. For preliminary experiments the duty cycle was split to scan for 5 antimalarials in each run. The antimalarials were sorted based on the compatibility of their respective ESI tune files; specifically they were sorted based on

whether they required a high or low tube lens offset. The gradient used for all preliminary experiments was 5 – 100% B ramped in 30 minutes. Once each antimalarial was detected, first the maximum injection time and AGC settings were adjusted to optimize the peaks. Next, all 10 antimalarials were combined into a single run, with the detection settings listed in Table IV.

Parameter	Setting
ISV	5
Sheath gas flow rate	14
Capillary voltage (V)	19.03
Capillary temp (C)	300
Tube lens (V, sp)	18.5
Injection time (ms)	50
# microscans	2
Collision energy (%)	36

Table IV: Tune file parameter settings for antimalarial mixture.

Once each antimalarial was detected with these settings, the HPLC flow and solvent gradient were optimized.

4.4.1 Flow Optimization

For flow optimization, the antimalarial mixture (5 μM) was run 5 times with different flow rates ranging from 200 - 700 μL/min. Although a flow rate of 600 μL/min produced the best full width at half maximum (FWHM) as seen in Table V for amodiaquine, the HPLC pumps over-pressured and failed at this flow rate or higher when the gradient consisted of mostly water. For this reason and because the optimal flow rate for the column is 500 μL/min, this flow rate was chosen for all further experiments.

Flow Rate (μL/min)	Retention Time (min)	Area	FWHM (min)
200	9.63	185194233	1.60
400	7.03	340325707	1.02
500	5.27	257120535	0.67
600	4.78	233182804	0.53
700	6.41	263114507	0.61

Table V: Chromatographic parameters for amodiaquine at five different flow rates using a 5 μM solution and 5-100% B gradient ramped in 30 minutes. (FWHM stands for full width at half maximum).

4.4.2 Gradient Optimization

Once the optimal flow rate was determined, the gradient parameters were adjusted to further optimize the chromatography and shorten the run time. The initial gradient used for experimentation was 5-100% B ramped in 30 minutes. Since all of the antimalarials did not elute until after a 20% B concentration was reached, the first trial gradient was started at a higher, 15%, initial B concentration ramped for the same time, 30 minutes. This successfully eluted all antimalarials; however it did not improve the throughput of the method. Next, the same % B concentration was used, only this time the program was ramped in 15 minutes. Finally, an even faster method of 20-100% B in 10 minutes was also tested. As seen in Table VI, this final gradient setting produced the best FWHM and shortest run time, while still successfully eluting each antimalarial. Therefore this gradient was chosen for all further experiments.

% B	Ramp time (min)	Retention Time (min)	Area	FWHM (min)
15-100	30	5.41	49520327	1.38
15-100	15	4.44	40126821	0.75
20-100	10	2.5	51004074	0.70

Table VI: Chromatographic parameters for amodiaquine with three different gradient programs, with a 500 μL/min flow rate and 1 μM solution.

4.4.3 Optimized Method

The optimized method therefore consisted of the tune file settings from Table IV, a 500 μL/min flow rate, and 20-100% B gradient ramped in 10 minutes for a total run time of 10 minutes. Table VII summarizes the chromatographic parameters including retention time (RT), area, and FWHM for each antimalarial and Figure V displays the mass selected chromatograms for each as well.

Antimalarial	Transition	Average RT	Average Peak Area	Average FWHM (min)
Amodiaquine	356.1 - 283.2	2.87 ± 0.74	4.45E+07 ± 38 %	0.79 ± 0.13
Chloroquine	320.1 - 247.2	2.77 ± 0.71	9.42E+07 ± 16.5 %	0.72 ± 0.14
Dapsone	249.1 - 156.0	4.46 ± 0.19	2.90E+07 ± 3.6 %	1.30 ± 0.09
Dihydroquinine	327.3 - 309.2	2.90 ± 0.58	4.36E+07 ± 33.8 %	1.15 ± 0.62
Mefloquine	379.2 - 361.2	5.85 ± 0.21	1.50E+08 ± 120.1 %	1.26 ± 0.13
Primaquine	260.1 - 243.2	4.12 ± 0.23	4.17E+07 ± 26 %	1.25 ± 0.27
Pyrimethamine	249.3 - 233.1	3.99 ± 0.22	6.88E+04 ± 13.4 %	0.50 ± 0.13
Quinine	325.3 - 307.2	2.95 ± 0.71	2.32E+07 ± 59.5 %	0.89 ± 0.22
Sulfadoxine	311.2 - 156.0	4.76 ± 0.11	4.41E+07 ± 13.4 %	1.12 ± 0.21
Trimethoprim	291.3 - 230.1	2.89 ± 0.67	1.20E+07 ± 6.5 %	0.68 ± 0.04

Table VII: The averaged chromatographic parameters for each antimalarial with the optimized LC-MS/MS method. Three trials were performed using a 1 μM solution with a 0.5 mL/min flow rate, 20-100% B gradient ramped in 10 minutes, and 10 minutes equilibration time between runs.

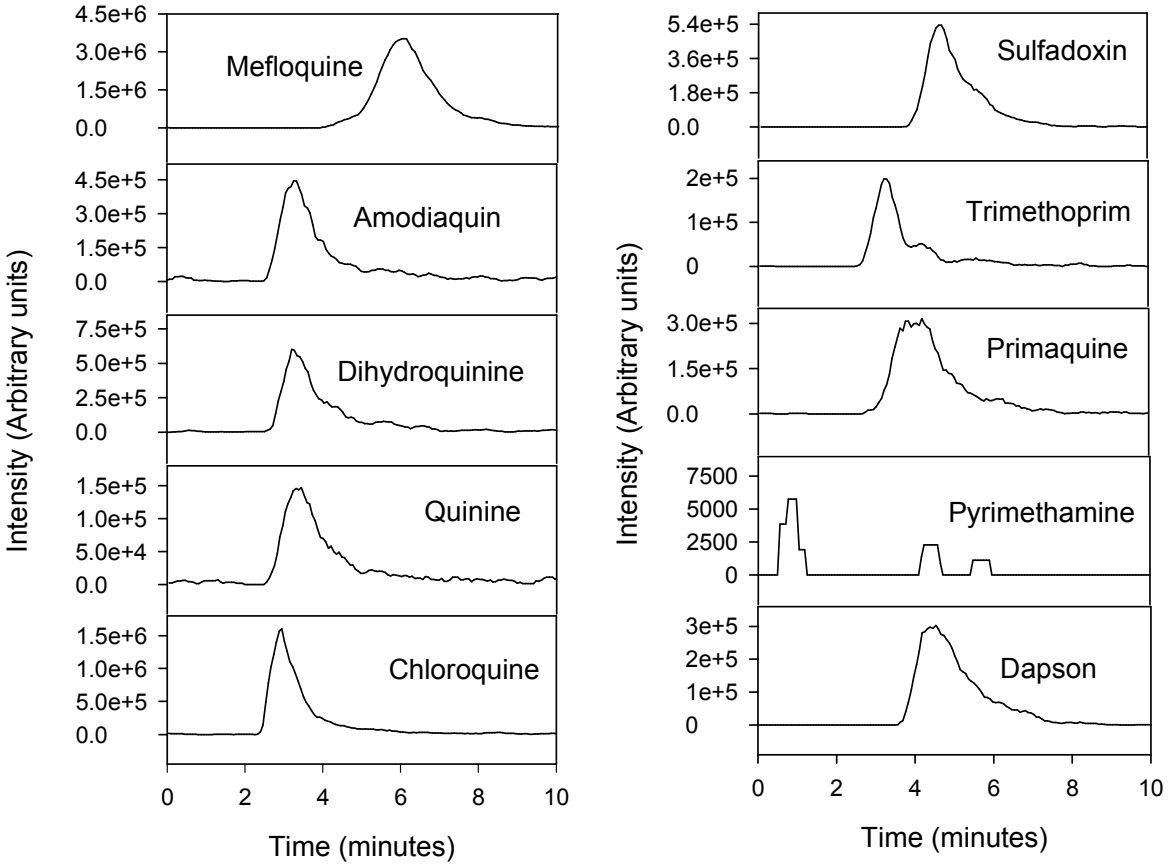


Figure V: The chromatograms for each antimalarial using the optimized LC-MS/MS method with a 1 μM sample solution, 0.5 mL/min flow rate, and 20-100% B gradient ramped in 10 minutes.

Each antimalarial was successfully detected with the optimized method. However, as evident in Figure V, the peak intensity for pyrimethamine was very low and was so consistently throughout all experiments. This was likely due to the very poor solubility of the drug, and the signal intensity could not be improved even with addition of acetonitrile or preparation of fresh stock solutions. Additionally, a large variability of peak area was observed for a number of antimalarials as seen in Table VII. Causes for this could

include inconsistencies with the pumps as the pressures during analysis oscillated irregularly, or a non-homogeneous sample mixture.

Next, antimalarial mixtures of four different concentrations, 0.1, 0.3, 0.5, and 1.0 μM, were run using the optimized method to produce a calibration curve for each antimalarial. The plot for each antimalarial, except for pyrimethamine which did not provide a strong signal at any concentration, and its corresponding linear regression and R^2 value can be seen in Figure VI. Regression parameters are also summarized in Table VIII.

Certain regression lines also did not intersect near the origin. Reasons for this can be attributed to the fact that peaks for some antimalarials appeared with a 0.1 μM concentration, whereas all of the other drugs were undetected with this concentration. Since these solutions were prepared by serial dilution from a 1 μM solution, it is possible that some 1.0 μM solutions were prepared improperly, or carryover from previous runs resulted in peaks with the 0.1 μM solutions since this was not the first concentration analyzed.

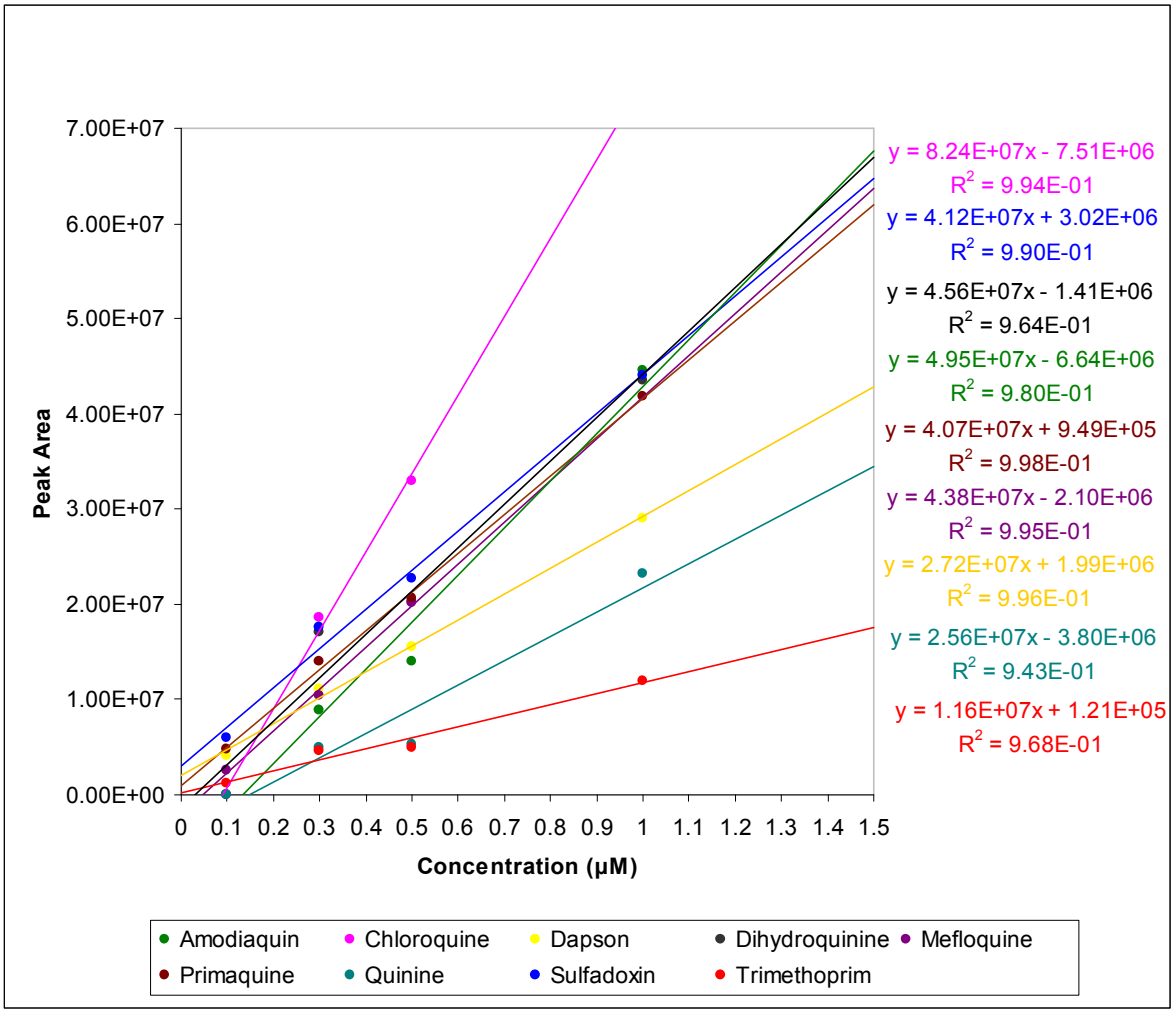


Figure VI: Calculated calibration curves for each antimalarial except pyrimethamine from 0.1, 0.3, 0.5, and 1.0 µM solutions.

Compound	Slope (m)	Intercept (b)	R^2
Amodiaquine	4.95E+07	-6.64E+06	0.980
Chloroquine	8.24E+07	-7.51E+06	0.994
Dapsone	2.72E+07	1.99E+06	0.996
Dihydroquinine	4.56E+07	-1.41E+06	0.964
Mefloquine	4.38E+07	-2.10E+06	0.995
Primaquine	4.07E+07	9.49E+05	0.998
Quinine	2.56E+07	-3.80E+06	0.943
Sulfadoxin	4.12E+07	3.02E+06	0.990
Trimethoprim	1.16E+07	1.21E+05	0.968

Table VIII: Regression data for antimalarial calibration curves.

The final step in method development was the preparation and analysis of control human plasma samples, each spiked with a different antimalarial. The identity of the antimalarial in each was unknown to the person performing the analysis. This tested the method's ability to identify a single antimalarial from a sample, and also gave an estimate to the quantitative abilities based on the developed calibration curves.

Three different plasma samples were prepared, and the antimalarial in each was successfully identified as chloroquine, amodiaquine, and dihydroquinine. The chromatograms from the chloroquine sample are shown in Figure VII. Although chloroquine was correctly identified, another peak, mefloquine, was also visible in the chromatograms as seen in Figure VII. However, based on mefloquine's peak intensity, retention time, and overall peak shape in comparison the chloroquine's peak, it was deduced that mefloquine was not the spiked antimalarial, and the peak was rather a result of carryover or contamination from previous runs. The remaining antimalarial chromatograms only contained noise.

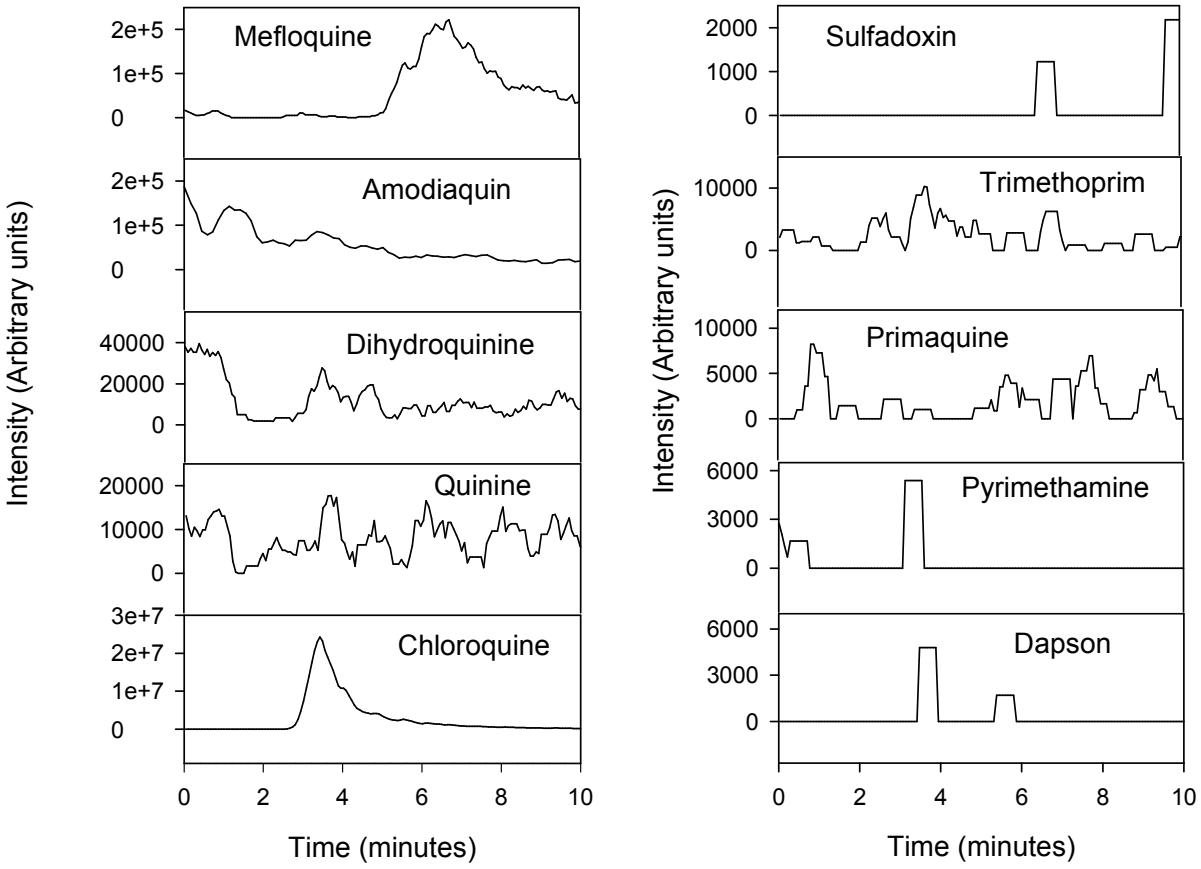


Figure VII: Chromatograms for each antimalarial from an unknown spiked plasma sample. The unknown antimalarial for this sample was identified as chloroquine, although a weak and possibly contaminant mefloquine peak was also present.

The concentration of each spiked antimalarial was also calculated using the resulting peak area and calibration curves. However, the calculated concentrations 17.0 μM, 0.77 μM, and 0.19 μM were far from the actual prepared concentrations (5 μM, 0.4 μM, and 0.6 μM, respectively). This may have been caused by either poor recovery of the antimalarial from the complex plasma matrix, or it may have been due to carryover between successive runs, indicating that the method will need further optimization before use to quantitate the amount of antimalarial present in a sample.

CHAPTER 5: CONCLUSIONS

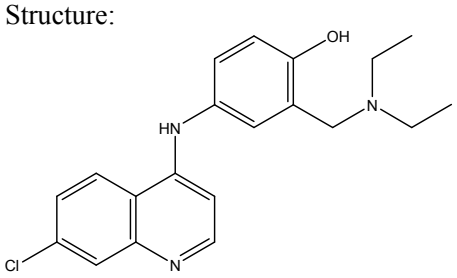
This preliminary method development using LC-MS/MS confirms that multiple unrelated antimalarial drugs can be successfully separated and detected with high sensitivity using this technique. Each antimalarial was successfully separated and detected, and with the exception of pyrimethamine, each had excellent peak intensity and shape. However, a limitation of this method is that although the Xcalibur software will allow for a maximum of 20 different screening transitions to be performed per run, the corresponding reduction in the duty cycle, or scan time for each transition, may significantly limit the sensitivity. Therefore in order to screen for additional antimalarial drugs using this method, either the chromatographic separation of these compounds needs to be improved to allow for the scanning of a few transitions at different times during the run (which is possible using this software) or the analysis needs to be performed as two sequential runs scanning for different compounds. Both suggested solutions to this problem will reduce the throughput of the method but as the method is already relatively short, and currently the analysis of 120 antimalarials can be performed per hour (20 antimalarials per run with a 10 minute run time), it is anticipated that the impact of these changes would be minimal.

Further work could also prepare the method for more accurate quantitative capabilities. Analysis of more samples using this approach, including the preparation and analysis of neat standards as well as standards prepared using complex biological matrices such as serum or plasma, would be beneficial for determining how accurate the

current quantitation actually is, and if the inaccuracies of the unknown plasma samples were due to the method, poor recovery of the drugs from the plasma, or carryover.

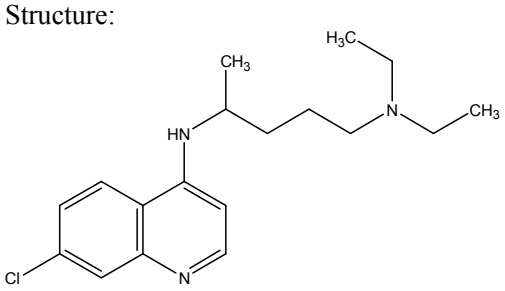
APPENDIX A: Antimalarial Structures

1. **Amodiaquine**
   Formula: $C_{20}H_{22}ClN_3O$
   Monoisotopic Mass: 355.15
   Structure:



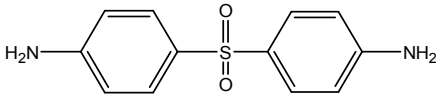
   Info: Also known as under the trade names Campoquin and Flavoquin. A 4-aminoquinoline compound related to chloroquine. It is used as an antimalarial and anti-inflammatory, and has been shown to be more effective than chloroquine in treating chloroquine-resistant malaria infections. The drug is not marketed in the U.S. but is widely available in Africa. The drug form amodiaquine hydrochloride is readily absorbed from the gastrointestinal tract where it is converted in the liver to the active metabolite desethylamodiaquine.

2. **Chloroquine**
   Formula: $C_{18}H_{26}ClN_3$
   Monoisotopic Mass: 319.18
   Structure:



   Info: Chloroquine phosphate is a 4-aminoquinoline compound that is an antimalarial and immune suppressant drug. It is known to have resistance to the *Plasmodium falciparum* strains. It is also being investigated as an antiretroviral drug. Chloroquine can be used for preventing malaria from *Plasmodium vivax, ovale* and *malariae*.

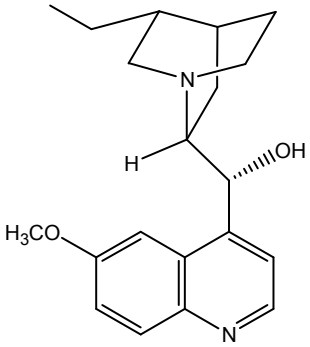
3. **Dapsone**
   Formula: $C_{12}H_{12}O_2N_2S$
   Monoisotopic Mass: 248.06
   Structure:



   Info: A drug used for malaria prevention that is commonly combined with other antimalarial preventatives. Like other sulpha drugs, dapsone inhibits dihydropteroate synthase in the malaria parasite.

4. **Dihydroquinine**

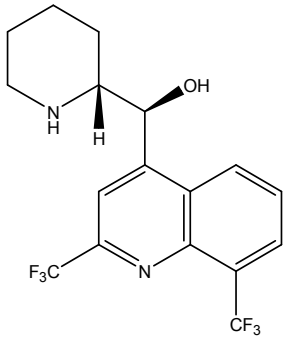
Formula: $C_{20}H_{26}N_2O_2$
Monoisotopic Mass: 326.20
Structure:



Info: Dihydroquinine, also known simply as hydroquinine, is an organic compound and is a cinchona alkaloid closely related to quinine.

5. **Mefloquine**

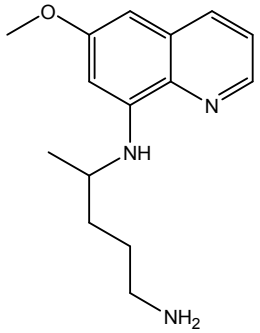
Formula: $C_{17}H_{16}F_6N_2O$
Monoisotopic Mass: 378.07
Structure:



Info: An antimalarial agent found in the hydrochloride form.

6. **Primaquine**

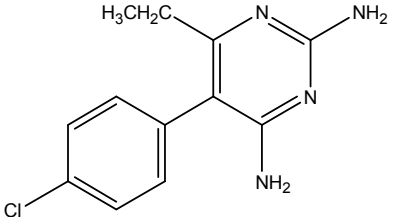
Formula: $C_{15}H_{21}N_3O$
Monoisotopic Mass: 259.17
Structure:



Info: Primaquine phosphate ($C_{15}H_{27}N_3O_9P_2$) is an 8-amino-quinoline compound which eliminates tissue infection. It also prevents the development of the blood forms of malaria which are responsible for relapses. Primaquine phosphate is also active against gametocytes of *Plasmodium falciparum*

7. **Pyrimethamine**

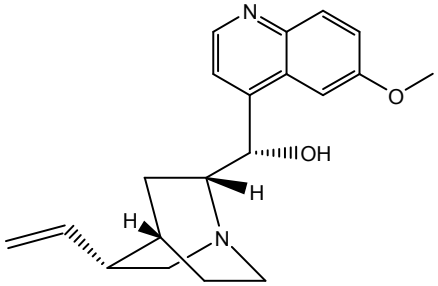
Formula: $C_{12}H_{13}ClN_4$
Monoisotopic Mass: 248.08
Structure:



Info: A component commonly found in the antimalarial drug Fansidar that is used in malaria preventatives and treatments. It is a folic acid antagonist that inhibits the activity of dihydrofolate reductase. It is active against the *Plasmodium falciparum* that are typically chloroquine resistant

8. **Quinidine**

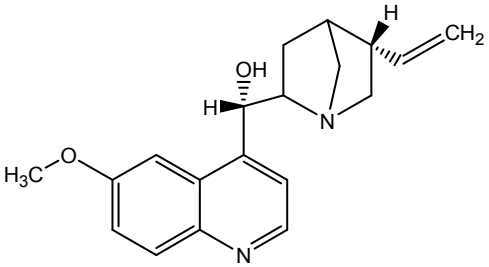
Formula: $C_{20}H_{24}N_2O_2$
Monoisotopic Mass: 324.18
Structure:



Info: Quinidine is an antimalarial schizonticide and an antiarrhythmic agent. Quinidine sulfate is the sulfate salt of quinidine and it has an additional H_2SO_4 * $2H_2O$ with its dimer structure. Intravenous quinidine is used for treatment of *P. falciparum* malaria.

9. **Quinine**

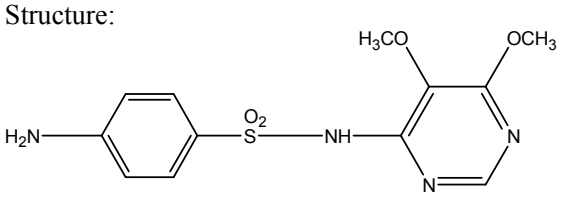
Formula: $C_{20}H_{24}N_2O_2$
Monoisotopic Mass: 324.18
Structure:



Info: Quinine was the first effective treatment for *falciparum malaria*, appearing in therapeutics in the 17th century. It remained the antimalarial drug of choice until the 1940s, when other drugs took over. Since then, many effective antimalarials have been introduced, although quinine is still used to treat the disease in certain critical situations. Quinine is available with a prescription in the United States. It is also used to treat nocturnal leg cramps and arthritis.

10. **Sulfadoxine**

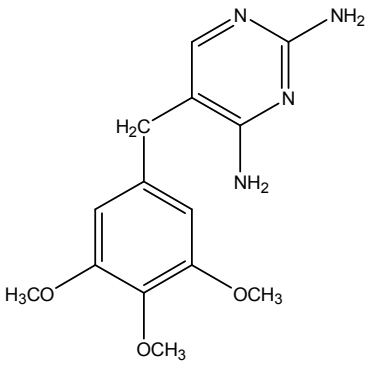
Formula: $C_{12}H_{14}N_4O_4S$
Monoisotopic Mass: 310.07
Structure:



Info: A component commonly found in the antimalarial drug Fansidar. It is a folic acid antagonist that inhibits the activity of dihydropteroate synthase. It is active against the *Plasmodium falciparum* that are typically chloroquine resistant.

11. **Trimethoprim**

Formula: $C_{14}H_{18}N_4O_3$
Monoisotopic Mass: 292.13
Structure:



Info: Trimethoprim was formerly marketed by GlaxoWellcome under trade names Proloprim, Monotrim and Triprim which have since been licensed to various generic pharmaceutical manufacturers. It is a bacteriostatic antibiotic commonly used to treat prophylaxis and prescribed for prevention of malaria.

APPENDIX B: Method Descriptions

Multiple Reaction Monitoring (MRM):

In single reaction monitoring (SRM) the mass spectrometer is set to scan for a specific ion produced by fragmentation of the corresponding precursor ion. This is accomplished by selecting the precursor mass of the compound for MS/MS fragmentation and then monitoring for a single fragment ion. Only ions formed by the cleavage of a particular precursor ion are detected and plotted. Thus, the SRM plot is highly sensitive and can be used to identify a given compound even in a complex mixture containing compounds with a similar precursor mass. Combining several SRM transitions into a single experiment, denoted as multiple reaction monitoring (MRM), provides the ability to simultaneously identify several components within a complex mixture. Therefore, MRM experiments can be used to provide more specific and sensitive analyses for each compound of interest as compared to conventional LC methods utilizing UV or diode array detection.

Electrospray Ionization (ESI):

Electrospray ionization (ESI) is a technique used in mass spectrometry to produce ions. In electrospray ionization, a liquid is pushed through a very small, charged and usually metal, capillary. This liquid contains the substance to be studied, the analyte, dissolved in a large amount of solvent, which is usually much more volatile than the analyte. The high voltage applied to the capillary induces charge separation in the liquid whereby charge of the opposite polarity to that of the applied voltage resides closest to the capillary surface while charge of the same polarity as the applied voltage exits the

capillary. Depending on the polarity of the applied voltage, either cationic or anionic species can be formed. As the liquid exits the capillary, repelling charges create an aerosol with the help of an uncharged carrier gas. The solvent then evaporates bringing analyte molecules closer together and thus causing more repulsion. This repeats until the solvent is entirely evaporated and the lone analyte ions can then enter the mass spectrometer.

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